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A TECHNIQUE FOR EXTRACTION OF INTACT MITOCHONDRIAL DNA MOLECULES FROM LARVAE OF SATURNIID MOTHS (LEPIDOPTERA: SATURNIIDAE) FOR USE IN TAXONOMIC STUDIES

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ABSTRACT. Analysis of mitochondrial DNA can yield information about evolutionary relationships. In this paper, a set of procedures for the extraction and analysis of mtDNA from saturniid moths is described. Restriction fragment length polymorphisms reveal differences in the mtDNA of the species investigated. The potential of this methodology to contribute to comparative studies of moth species is discussed.

Additional key words: *Hyalophora*, *Callosamia*, RFLP, taxonomy, phylogeny.

The use of mitochondrial DNA (mtDNA) in studies of taxonomic and evolutionary relationships in insect groups has become well established since Bultman and Laird (1973) extracted, purified and described the physical characteristics of mtDNA from *Drosophila melanogaster* Meigen. Brower and Boyce (1991), Sperling (1993), Sperling and Harrison (1994), and Brower's (1994) extensive taxonomic and phylogenetic studies on butterflies are examples of the application of mtDNA organization to phylogenetic studies of Lepidoptera.

I became interested in carrying out mtDNA studies and began to learn the basic techniques of mtDNA extraction, purification and analysis. I chose to work with the genus *Hyalophora* Duncan (Lepidoptera: Saturniidae) because I had been rearing and studying *H. cecropia* (L) and *H. columbia gloveri* Strecker for many years. The several species, subspecies and hybrid populations of the genus occur in geographic areas that are distinct from each other but overlap (Scriber & Grabstein 1991, Collins 1997). They also show food preferences and thus occupy slightly different niches (Oberfoell 1969, Scriber & Grabstein 1991, Collins 1984).

The genus *Hyalophora* is presently considered to consist of three species: *H. cecropia* L., *H. columbia* Smith with the subspecies *H. columbia gloveri* Strecker and *H. columbia columbia* Smith; and *H. euryalus* Boisduval with one subspecies, *H. euryalus cedrosensis* Cockerell. This last subspecies, found only on the Isla de Cedros, Baja California, Mexico, was thought to be extinct until rediscovered and described by Smith and Wells (1993).

This organization of the genus was developed by Lemaire (1978) and reflects the studies of zones of hybridization between *H. euryalus* and *H. columbia gloveri* carried out by Sweadner (1937), repeated and extended by Collins (1973) and Kohalmi and Moens (1975, 1988). It is the classification used by Tuskes et al. (1996) and by Collins (1997).

Molecular evolutionary studies of saturniids include those of Collins et al. (1993) whose analysis of the distribution of 20 allozymes confirmed the hybrid nature of populations where *H. euryalus* and *H. columbia gloveri* came in contact with each other. Legge (1993) used DNA primers for the cytochrome oxidase II (COII) gene, in conjunction with polymerase chain reaction (PCR) amplification, to isolate COII genes form total genomic DNA of several species of Hemileuca. Legge (1993) was able to use the nucleotide sequences of COII genes to construct consensus trees for phylo-

genetic analysis of species of the genus *Hemileuca*. Friedlander et al. (1998) used the sequence information of two nuclear genes to construct consensus trees showing the phylogenetic relationships of species of the Attacini, including the three *Hyalophora* species, and the Saturniini.

To my knowledge, no one has yet reported the isolation of complete mtDNA molecules from Saturniid species. Having the complete molecule would be an asset to anyone investigating phylogenetic relationships between moth species. It turns out that the moth tissue having the greatest quantity of mitochondria is the gut of mature larvae. Anderson and Harvey (1966) studied the fine structure of the *H. cecropia* midgut epithelium and found that both the microvillae which extended into the gut lumen and the channels formed by the deep infoldings of the apical and basal plasma membrane were packed with mitochondria. This rich source of mitochondria was used to initiate a comparative study of the mtDNA of *Hyalophora* species. In this paper I present the methodology developed and some initial results of this study.

MATERIALS AND METHODS

All glassware, Eppendorf tubes, pipet tips and solutions are autoclaved prior to mtDNA processing. Items that are going to be reused are washed in hot water containing Alconox ™ detergent, then rinsed in tap water, followed by a rinse in deionized water. These are air dried, placed in glass containers which are capped with aluminum foil, and autoclaved for 30 min at 120°C. Plastic gloves are worn during extraction procedures to reduce the chance for contamination of the mtDNA.

Mitochondria are taken from the guts of healthy 4th or 5th instar larvae which have been cleaned of their contents. The larvae used for this study were reared from ova deposited by 15 *H. cecropia*, 8 *H. columbia gloveri*, 2 *H. columbia columbia*, 7 *H. euryalus*, and 2 *Callosamia promethea* Drury. The last were used as an outgroup. The sources of ova are listed in Table 1.

Larvae, except as noted below, are laboratory reared in plastic sweater boxes on fresh leaves of cherry (*Prunus* sp.) or white oak (*Quercus alba* L.) which have been sprayed with an antibiotic solution (Riddiford 1967). The number of larvae per box is kept small and larvae are handled as little as possible. *H. columbia* are reared on branches of *Larix laricina* (DuRoi) K. Koch.

Mature larvae are anesthetized under carbon dioxide gas, decapitated and severed near the posterior. The body wall is cut through along the ventral side and the larva is pinned out. Next, the gut is cut longitudinally along its ventral side and the gut contents are removed. In the best of cases, the peritrophic membrane can be lifted and rolled forward to remove the gut contents cleanly. The tracheal trunks that serve the gut are severed. The gut is removed from the body cavity, washed thoroughly in deionized water and transferred to containers chilled in ice or dry ice. Most tissues are weighed and stored at –70°C within the hour. Each sample consists of 4 or 5 guts. These samples are processed to extract mtDNA as soon as possible. Tissues that are to be processed immediately are transferred to a chilled Wheaton dounce homogenizer which contains 5 ml of a physiological buffer (0.44 M sucrose, 0.01 M Tris, 0.18 mM EDTA; pH 7.5). Tissues in the dounce, either fresh or frozen, are macerated and the mtDNA is extracted using a procedure described by Tamura and Aotsuka (1988), with the modifications given below.

TABLE 1. Suppliers of the ova used in this research.

Moth species	Supplier
H. cecropia	+ Ralph M. Clark, Plattsburgh, NY
	Larry Kopp, Klingerstown, PA
	+ * James Oberfoell, Bowman, ND
	Mark Schmidt, Springboro, OH
	Scott Smith, Santa Rosa, CA
H. euryalus	+ Dean Morewood, Victoria, BC, Canada
	+ Norman Smith, Fresno, CA
	Scott Smith, Santa Rosa, CA
H. columbia columbia	Gardiner Gregory, Orland, ME
	William Kenny, Dixemont, ME
	° Mark Schmidt, Springboro, OH
	+ Ted Herig
H. columbia gloveri	+ * James Oberfoell, Bowman, ND
	Mark Schmidt, Springboro, OH
	Scott Smith, Santa Rosa, CA
A. polyphemus	Mark Schmidt, Springboro, OH
C. promethea	Larry Kopp, Klingerstown, PA
	Mark Schmidt, Springboro, OH

+ Native moths reared by supplier from representatives of local populations.
* Present address unknown.
° From stock supplied by W. Kenny.

Differential centrifugation in a physiological buffer is followed by alkaline lysis of the mitochondrial fraction. A centrifugation step separates the mtDNA from cellular debris and the mtDNA is further purified by phenol extraction. The mtDNA is precipitated from an alcoholic solution, resuspended in $T_{10}E_1$ and treated with the enzyme, RNase, before storage at –20°C.

Standard endonuclease digestions are carried out using a variety of endonucleases. A digest of lambda DNA with Hind III is used to generate a reference kilobase pair (Kbp) ladder against which to size restriction fragments. The digests are loaded into the wells of 1% agarose (GIBCO/BRL) gels. Electrophoresis is carried out in a Hoefer Scientific Instru-

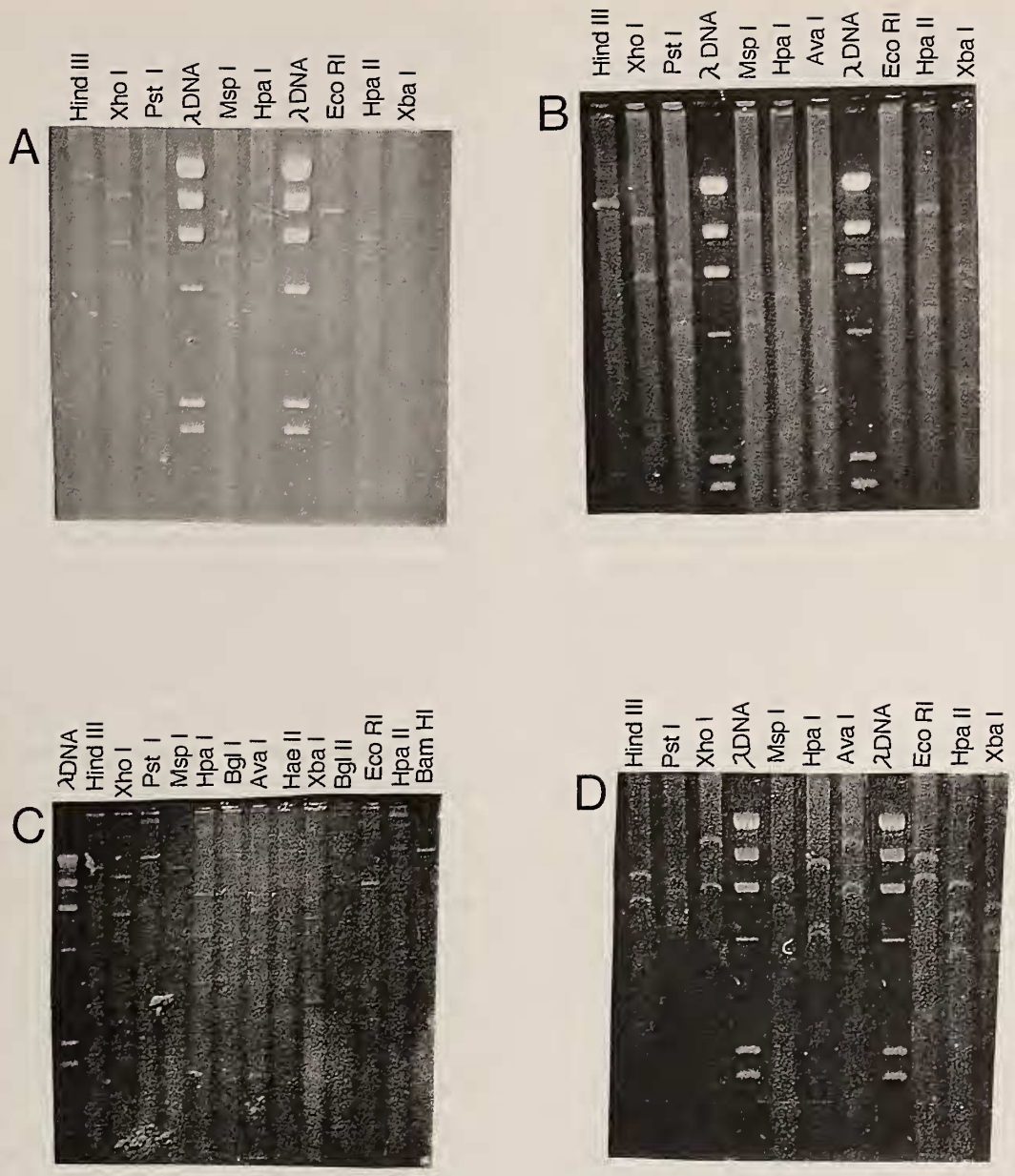

FIG. 1. Representative electrophoretic patterns for A) *H. columbia columbia*; B) *H. euryalus*; C) *H. cecropia*; and D) *C. promethea*.

ments unit set at 37V, 23 milliamps. TBE is the electrophoretic buffer. The gel slab is stained with ethidium bromide, then destained in 0.1 mM $MgSO_4$ before examination under ultraviolet light. Gels are photographed and common pins are positioned in the gel to mark the position of mtDNA fragments. Measures of distances of the pins from the wells are taken and used to construct gel replicas and to determine the sizes of the mtDNA fragments in kilobase pairs (Kbp).

RESULTS

The photographs in Fig. 1a, b, c, d are examples of electrophoretic patterns obtained when mtDNA from larvel guts is digested with these endonucleases. A number of bands the size of the largest λ-DNA fragment or larger is seen in Fig. 1c. These are taken as evidence of incomplete digestion; the presence of non-mt-DNA in the sample, and/or from complexes

TABLE 2. Number of endonuclease sites observed in mtDNA of four saturniid species for the listed enzymes.

Species	Enzyme			
	Hind III	Msp I (Hpa II)	Hpa I	Pst I
H. cecropia	3	2	3	2
H. columbia	3	3	3	3
H. euryalus	3	2	2	3
C. promethea	2	3	3	3

TABLE 3. Haplotypes of species of *Hyalophora* and *C. promethea* based on electrophoretic patterns after digestion of mtDNA with the listed enzymes. *H. cecropia*'s haplotype is used as the basis for comparison. RFLPs are indicated by letter changes. The two sub-species of *H. columbia* exhibit identical haplotypes.

Species	Enzyme						
	Hind III	Msp I (Hpa II)	Hpa I	Pst I	Ava I	Xho I	EcoRI
H. cecropia	A	A	A	A	A	A	A
H. columbia	A	B	A	B	A	A	A
H. euryalus	A	A	B	B	A	A	A
C. promethea	B	B	C	B	A	B	B

formed when the DNA concentration in a sample is excessive.

The patterns of cleavage for these species are very consistent. Not shown is the pattern for *H. columbia gloveri* which is identical to *H. columbia columbia*. Eight of the endonucleases tested to date yield information useful for phylogenetic analysis. These are Hind III, XhoI, PstI, MspI, EcoRI, AvaI, HpaI and HpaII. The isoschizomers MspI and HpaII have identical cleavage patterns indicating that the cytosines of 5-CCGG-3 sequences are not methylated. XbaI results are very inconsistent. This enzyme is known for aberrant cleavage if exact reaction conditions are not followed (Gibco/BRL 1997–98 Catalog). Among the endonucleases that do not cleave these saturniid mtDNAs are BglI, HaeII, BglII and Bam HI (Fig. 1c).

Species specific differences in cleavage patterns are observed when the photographs in Fig. 1 are compared (Table 2). There are no observed differences in the number of cleavage sites for the other endonucleases. The two small Hind III cleavage sites are about 2.2 and 1.7 kilobase pairs (Kbp) long and difficult to see as they stain faintly with ethidium bromide, and photograph poorly.

The single band seen when *Hyalophora* mtDNA is cut by EcoRI (Figs. 1a, b, c) is judged to be about 920 Kbp long, based on its position relative to the 960 Kbp band of the λ-Hind III standard. Since all my results indicate that moth mtDNA is between 14–19 Kbp in length (a size range common to animals), EcoRI apparently cuts the mtDNA in two places which yields two fragments of approximately equal size. The two fragments comigrate. The *C. promethea* mtDNA is also cut at two locations by Eco RI, but the product is two fragments of unequal size, thus there are two bands (Fig. 1d). The size of these two fragments totals to about 16 Kbp. This is one of two examples seen where RFLPs result from an internal reorganization of mtDNA, not from the gain or loss of the number of enzyme sites. A second example is seen when the HpaI digest pattern of *C. promethea* is compared to the hyalophorans (Fig. 1).

Table 3 presents the haplotypes of the species studied, based on RFLPs. If one elects the *H. cecropia* haplotype as the base against which to compare the others, then one can see that the *H. cecropia* restriction pattern differs from *H. columbia* and *H. euryalus* for two of seven enzymes, but not the same enzymes; from the *C. promethea* haplotype for 5 of 7 enzymes.

H. columbia haplotypes show two mtDNA modifications from *H. euryalus* and three from *C. promethea*. *H. euryalus* differs from *C. promethea* at five sites, but not the same five by which *C. promethea* and *H. cecropia* differ. The PstI digest pattern of *H. cecropia* is unique.

Most of these polymorphisms are caused by the gain or loss of sites for enzyme attack, however the uniqueness of the *C. promethea* haplotype may result from RFLPs created by internal rearrangements of the mtDNA (HpaI and EcoRI digests).

DISCUSSION

The major reason for this paper is to present procedures for the extraction, partial purification and analysis of complete molecules of moth mtDNA. This has been accomplished by adapting procedures of others to the extraction of mtDNA from the guts of mature moth larvae. The results show that larval lepidoptera are good sources of mitochondrial DNA for use in studies of taxonomy and phylogeny. Even though the mtDNA obtained using this methodology was not highly purified, and even though the electrophoretic stain used was not the most sensitive, results are quite reliable. Distinct RFLPs and haplotypes of the four saturniid species were observed.

Contamination of the mtDNA with nuclear DNA was not a problem. Tamura and Aotsuka (1988) stated that the purity of mitochondria as a result of differential centrifugation is not important because the alkaline lysis procedure efficiently separates covalently closed circular mtDNA from linear DNA. Legges' (1993) concern that mtDNA from gut tissues could be seriously contaminated by gut organisms are allayed by

the results presented here. By using the procedures of Jones et al. (1988) greatly improved purification and fragment resolution is possible.

The advantage of this method over that utilized by Legge (1993) and others to obtain and sequence the CoII gene is that the entire mtDNA molecule is obtained. This allows studies of RFLPs and the construction of restriction maps of the mtDNA chromosome based on double-digest studies. Additionally, sequencing of entire mtDNA molecules is possible. The results of such studies will reveal any internal rearrangement or mutations of the mtDNA molecule that is species or population specific. Two such internal rearrangements have been discovered during this study (Table 3). Sequencing of the entire molecule may reveal any introgression of genetic sequences were hybrid zones exist. Kondo et al. (1990) presented clear evidence of heteroplasmy in *Drosophila* and the occurrence of introgression of mtDNA. Introgression of mtDNA in *Drosophila* was also observed by Aubert and Solignac (1990). If introgression is found in natural hybrid zones it could reveal cross-overs between native and introduced mtDNAs and help clarify evolutionary relationships within *Hyalophora*. It could also reveal the existence or establishment of subpopulations within a population by revealing two or more maternal lineages that are established through reciprocal crosses.

Table 3 is based on a system used by Avise and Nelson (1989) to illustrate relationships between the genomes of seaside sparrows that were dispersed over a wide geographic area of the southeastern United States. I have adapted this system as it seems to illustrate haplotype differences most clearly.

The distinctness of the *C. promethea* mtDNA from that of *Hyalophora* is readily apparent as 3 of the 7 enzymes used to digest the samples yield results that are unique to *C. promethea*. This pattern would suggest a separate line of evolution for the *Callosamia* and would corroborate the findings of Friedlander, et al. (1998) and Johnson, et al. (1996). One can infer that the identity of the digest patterns of the two subspecies of *H. columbia* indicates that these two are more closely related to each other than they are to the other *Hyalophora*. Also, the PstI digest pattern which is unique to *H. cecropia* suggests an evolutionary separation from the others, with the PstI pattern of *C. promethea* possibly being more ancient and unchanged in columbians and euryalus. However one must be aware that both forward and reverse mutations occur.

There is not enough information here to make further inferences as to the degree of relatedness and lines of evolution of the *Hyalophora*. However, nothing in these results contradict the findings of Sweadner (1937), Lemaire (1978), Tuskes, et al. (1996) or Collins (1973, 1997). It would be interesting to have samples of *H. euryalus cedroensis* mtDNA to study because of its potentially long-time isolation from the rest of the *Hyalophora*. The naming of the two *H. columbia* subspecies creates confusion because even though *H. columbia gloveri* is believed to an ancestral to *H. columbia columbia* (Sweadner 1937, Collins 1997), the *H. columbia* name has priority.

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LITERATURE CITED

ANDERSON, E. A. & W. R. HARVEY. 1966. Active transport by the Cecropia midgut. II. Fine structure of the midgut epithelium. J. Cell. Biol. 31:107–134.

AUBERT, J. & M. SOLIGNAC. 1990. Experimental evidence for mitochondrial DNA introgression between *Drosophila* species. Evolution 44:1272–1282.

AVISE, J. C. & W. S. NELSON. 1989. Molecular genetic relationships of the extinct dusky seaside sparrow. Science 243:646–648.

BROWER, A. V. Z. 1994. Rapid morphological radiation and convergence among races of the butterfly *Heliconius erato* inferred from patterns of mitochondrial DNA evolution. Proc. Nat. Acad. Sci. 91:6491–6495.

BROWER, A. V. Z. & T. M. BOYCE. 1991. Mitochondrial DNA variation in monarch butterflies. Evolution 45:1281–1286.

BULTMAN, H. & C. D. LAIRD. 1973. Mitochondrial DNA from *Drosophila melanogaster*. Biochem. Biophys. Acta 299:196–209.

COLLINS, M. M. 1973. Notes on the taxonomic status of *Hyalophora columbia* (Saturniidae). J. Lepid. Soc. 27:225–235.

———. 1984. Genetics and ecology of a hybrid zone in *Hyalophora* (Lepidoptera: Saturniidae). U. Ca. Pub. in Entomology 104:1–93.

———. 1997. Hybridization and speciation in *Hyalophora* (Insecta: Lepidoptera: Saturniidae): a reappraisal of W. R. Sweadner's classic study of a hybrid zone. Ann. Carnegie Museum 66:411–455.

COLLINS, M. M., H. B. BRITTEN & V. RIVERS. 1993. Allozyme analysis of a known hybrid zone between *Hyalophora euryalus* and *H. columbia gloveri* (Lepidoptera: Saturniidae) in the California Sierra Nevada. J. Res. Lepid. 32:79–88.

FRIEDLANDER, T. P., K. R. HORST, J. C. REGIER, C. MITTER, R. S. PEIGLER & Q. Q. FANG. 1998. Two nuclear genes yield concordant relationships within Attacini (Lepidoptera: Saturniidae). Molec. Phylog. Evol. 9:131–148.

JOHNSON, K. S., D. SNIDER & J. M. SCRIBER. 1996. Estimates of genetic differentiation among *Callosamia* species and *Hyalophora cecropia* (Saturniidae) using allozyme electrophoresis. J. Lepid. Soc. 50:217–225.

JONES, C. S., H. TEGELSTROM, D. S. LATCHMAN & R. J. BERRY. 1988. An improved rapid method for mitochondrial DNA isolation suitable for use in the study of closely related populations. Biochem. Gen. 26:83–88.

KOHALMI, L., & P. MOENS. 1975. Evidence for the existence of an intergrade population between *Hyalophora gloveri nokomis* and *H. columbia* in Northwestern Ontario (Lepidoptera: Saturniidae). Can. Entomol. 107:793–799.

———. 1988. Patterns of inheritance of larval tubercle-colour polymorphism in *Hyalophora columbia* of northwestern Ontario (Lepidoptera: Saturniidae). Genome 30:307–312.

KONDO, R., Y. SATTA, E. T. MATSUURA, H. ISHIWA, N. TAKAHATA & S. I. CHIGUSA. 1990. Incomplete maternal transmission of mitochondrial DNA in Drosophila. Genetics 126:657–663.

LEGGE, J. T. 1993. Genetic differentiation of fen-dwelling *Hemileuca* (Lepidoptera: Saturniidae) within the *H. maia* species group. Master's Thesis, Cornell University Graduate School, Ithaca, New York. 121 pp.

LEMAIRE, C. 1978. Les Attacidae Americains. Attacinae. Edition C. Lemaire, Neuilly-sur-Seine, France. 238 pp.

OBERFOELL, J. 1969. Occurrence of the cecropia and Rocky Mountain silk moths in southwestern North Dakota. Prairie Natur. 1:63–64.

RIDDIFORD, L. M. 1967. Antibiotics in the laboratory rearing of Cecropia silkworms. Science 157:1451–1452.

SCRIBER, J. M. & E. GRABSTEIN. 1991. Food plant processing adaptations in four *Hyalophora* species (Lepidoptera: Saturniidae): regional and taxonomic specialization. Great Lakes Entomol. 24:109–120.

SMITH, M. J. & R. E. WELLS. 1993. Rediscovery of *Hyalophora euryalus cedrosensis* (Saturniidae), with descriptions of the adult and larval stages. J. Lepid. Soc. 47:114–124.

SPERLING, F. A. H. 1993. Mitochondrial DNA variation and Haldane's rule in the *Papilio glaucus* and *P. troilus* species groups. Heredity 71:227–233.

SPERLING, F. A. H. & R. G. HARRISON. 1994. Mitochondrial DNA variation within and between species of the *Papilio machaon* group of swallowtail butterflies. Evolution 48:408–422.

SWEADNER, W. R. 1937. Hybridization and the phylogeny of the genus *Platysamia*. Ann. Carn. Mus. 25:163–243.

TAMURA, K. & T. AOTSUKA. 1988. Rapid isolation method of animal mitochondrial DNA by the alkaline lysis procedure. Biochem. Gen. 26:815–819.

TUSKES, P. M., J. P. TUTTLE & M. M. COLLINS. 1996. The wild silk moths of North America: a natural history of the Saturniidae of the United States and Canada. Cornell U. Press, Ithaca, New York. 250 pp.

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